AVIAN DISEASES 54:1086–1090, 2010

Research Note—

Surveillance and Identification of Influenza A Viruses in Wild Aquatic Birds in the Crimea, Ukraine (2006–2008)

M. V. Kulak,[A] F. A. Ilinykh,[A] A. V. Zaykovskaya,[A] A. V. Epanchinzeva,[A] I. L. Evstaphiev,[B] N. N. Tovtunec,[B] K. A. Sharshov,[A] A. G. Durimanov,[A] N. A. Penkovskaya,[B] A. M. Shestopalov,[A] I. Lerman,[C] I. G. Drozdov,[A] and D. E. Swayne[DE]

[A]State Research Center of Virology and Biotechnology "Vector," Department of Zoonotic Infection and Influenza, Novosibirsk region, Koltsovo, Russia 633159
[B]The Crimean Republican Sanitary Epidemiological Station, Crimea, Ukraine 98600
[C]Center for Integration of Medicine and Innovative Technology, Cambridge, MA 02139
[D]Exotic and Emerging Avian Viral Diseases Research Unit, Agricultural Research Service, USDA, Athens, GA 30605

Received 8 February 2010; Accepted and published ahead of print 6 April 2010

SUMMARY. The ecology of avian influenza (AI) viruses in wild aquatic birds of Asia is poorly understood, especially for the H5N1 high pathogenicity AI (HPAI) viruses. From March 2006 through November 2008, 20 AI viruses were isolated in the Crimea region of Ukraine with an overall frequency of virus recovery of 3.3%. All the viruses were isolated from three species of dabbling ducks: mallard (*Anas platyrhynchos*), wigeon (*Anas penelope*), and garganey (*Anas querquedula*), making the frequency of virus recovery for dabbling ducks 6.3%. The viruses were predominantly isolated during the fall sampling period. All viruses were genetically and antigenically characterized. No H5N1 HPAI viruses were isolated, but other HA and NA subtypes were identified including H3N1 (2), H3N6 (3), H3N8 (4), H4N6 (6), H5N2 (3), H7N8 (1), and H10N6 (1) subtypes. All isolates were of low pathogenicity, as determined by the intravenous pathogenicity index of 0.00. For H5N2 and H7N8 isolates, the HA gene was sequenced and the phylogenetic analysis revealed possible ecologic connections of the Crimea region with AI viruses from Siberia and Europe. No influenza A isolates were recovered from other Anseriformes (diving ducks [two species of pochards] and graylag geese), Columbiformes (collared doves), Gruiformes (coot), and Galliformes (gray partridges).

RESUMEN. *Nota de Investigación*—Vigilancia e identificación del virus de la influenza A en aves acuáticas silvestres en Crimea, Ucrania (2006–2008).

Se conoce poco acerca de la ecología de los virus de la influenza aviar en aves acuáticas silvestres en Asia, sobre todo para el virus de influenza aviar H5N1 de alta patogenicidad. A partir de marzo del 2006 hasta noviembre del 2008, veinte virus de influenza aviar han sido aislados en la región de Crimea en Ucrania, con una frecuencia general de recuperación de virus del 3.3%. Todos los virus fueron aislados de tres especies de patos chapoteadores: ánade real (*Anas platyrhynchos*), ánade silbón europeo (*Anas penelope*), y cerceta carretona (*Anas querquedula*), lo que contribuyó a observar una frecuencia de recuperación del virus en los patos chapoteadotes del 6.3%. Los virus fueron aislados principalmente durante el periodo de muestreo de otoño. Todos los virus fueron caracterizados genéticamente y antigénicamente. No se aislaron virus de la influenza aviar H5N1 de alta patogenicidad, pero se identificaron otros subtipos de HA y NA tales como H3N1 (2), H3N6 (3), H3N8 (4), H4N6 (6), H5N2 (3), H7N8 (1), y H10N6 (1). Todos los aislamientos fueron de baja patogenicidad, tal como se determinó por el índice de patogenicidad intravenosa de 0.00. Con relación a los aislamientos H5N2 y H7N8, se secuenció el gen HA y el análisis filogenético reveló posibles conexiones ecológicas entre los virus de la región de Crimea y los virus de influenza aviar de Siberia y Europa. No se aislaron virus de la influenza A de otros Anseriformes (patos buceadores [dos especies de porrones] y gansos comunes), Columbiformes (palomas de collar), Gruiformes (gallareta común), y Galliformes (perdices grises).

Key words: AI, Crimea, pathogenicity, surveillance, wild waterfowl

Abbreviations: AI = avian influenza; HA = hemagglutinin; HI = hemagglutination inhibition; HPAI = high pathogenicity AI; IVPI = intravenous pathogenicity index; LPAI = low pathogenicity AI; NA = neuraminidase; OIE = World Organisation for Animal Health; PBS = phosphate-buffered saline; RT-PCR = reverse transcriptase–polymerase chain reaction

Avian influenza (AI) is a highly contagious infection caused by type A influenza viruses, which are in the family *Orthomyxoviridae* (21). The type A influenza viruses are divided into subtypes based on the two surface glycoproteins, the HA and the NA. There are 16 (H1–16) HA and nine (N1–9) NA subtypes with a maximum of 144 possible combinations of HA:NA subtypes. Because influenza A viruses have a segmented genome, co-infection of one cell can result in an exchange of segments, leading to a diversity of reassortant strains and the emergence of new virus variants.

[E]Corresponding author. E-mail: David.Swayne@ars.usda.gov

Wild aquatic birds are the main reservoir of influenza A virus gene segments in nature and play a central epidemiologic role in the transfer of low pathogenicity AI (LPAI) viruses to domestic poultry. Ultimately, these wild bird AI viruses are the source of new gene segments to influenza A viruses in various mammals, including man. The vast majority of LPAI viruses do not cause disease in infected, wild aquatic birds, and there has been only rare isolation of high pathogenicity AI (HPAI) viruses from wild aquatic birds, most recently of H5N1 HPAI viruses in Asia (3). Furthermore, the ability of some H5 and H7 LPAI viruses to mutate and become of high pathogenicity in poultry raises additional concerns about the role wild birds may play in spreading both LPAI and HPAI viruses of H5

Table 1. Number of avian influenza viruses isolated from wild waterfowl in Crimea, Ukraine and the HA and NA subtype determinations.

Birds sampled	November 2006	March 2007	September 2007	March 2008	September 2008	No. of samples (%)
Mallard, *Anas platyrhyncha*	3/39[A] (H3N8, H4N6, H7N8)	0/2	0/0	0/1	5/60 (H3N1, H3N8, H4N6 [2], H5N2)	8/102 (7.8)
Wigeon, *Anas penelope*	1/9 (H3N8)	1/13 (H4N6)	0/11	0/4	0/0	2/37 (5.4)
Garganey, *Anas querquedula*	0/38	0/3	0/6	0/8	10/102 (H3N6 [3], H5N2 [2], H4N6 [2], H10N6, H3N1)	10/157 (6.4)
Northern shoveler, *Anas clypeata*	0/7	0/5	0/3	0/2	0/4	0/21
Pochard, *Aythya ferina*	0/6	0/0	0/18	0/23	0/92	0/139
Red-crested pochard, *Netta rufina*	0/4	0/5	0/8	0/3	0/11	0/31
Coot, *Fulica atra*	0/1	0/6	0/5	0/2	0/4	0/18
Collared dove, *Streptopelia decaocto*	0/11	0/8	0/2	0/3	0/21	0/45
Gray partridge, *Perdix perdix*	0/4	0/4	0/3	0/0	0/18	0/29
Greylag goose, *Anser anser*	0/1	0/13	0/3	0/10	0/0	0/27
All samples	4/120 (3.3)	1/59 (1.7)	0/59	0/56	15/ 312 (4.8)	20/606 (3.3)

[A]Positive/total samples (HA and NA subtype of isolates).

and H7 subtypes. Therefore, it is important to understand the ecology of influenza infection in wild aquatic bird populations (23).

Three general aquatic bird migration routes have the potential to include the territory of Crimea, Ukraine: the East Africa–West Asia, Central Asia, and Black Sea–Mediterranean flyways (13). The Crimea is used by numerous bird species as either a site of summer nesting or a stopover point during migration. For this reason, in the present paper we report the results of a survey on AI viruses recovered from hunter-killed migratory and nonmigratory wild birds within shared ecosystems in Crimea. The periods of sampling were all after the last H5N1 HPAI outbreak in 2005, which is described elsewhere (14).

MATERIALS AND METHODS

Surveillance area and sampling. Crimea is an autonomous republic of Ukraine located on the northern coast of the Black Sea and on the western coast of the Sea of Azov, occupying a peninsula of the same name. Crimea's total land area is 26,100 km^2 (10,038 mi^2). Crimea was the site of the last H5N1 HPAI outbreak in the Ukraine during 2005. From March 2006 through September 2008, a total of 606 cloacal swabs were collected from available hunter-killed migratory and nonmigratory wild birds within the same ecosystems. Hunting was conducted in four administrative areas: the Dzhankoy, Leninsky, Sovetsky, and Nizhnyohirsky regions. These areas were selected because they are bird congregation points during migration and they are regions where hunting is allowed. Other regions of the Crimea are mountainous and are not stopover points during migration. The cloacal swabs were collected in vials containing 0.8 ml phosphate-buffered saline (PBS):glycerol (1:1) with penicillin 40,000 U/ml, streptomycin 20 mg/ml, and kanamycin 20 mg/ml and immediately frozen and stored at −196 C (in liquid nitrogen) until virus isolation was attempted.

Virus isolation. Cloacal swabs were tested for influenza viruses by inoculation into the allantoic cavity of 10-day-old embryonating specific-pathogen-free chicken eggs according to standard procedures (25). Each sample underwent at least three passages in chicken eggs, and influenza isolates were identified by both HA assay (24) and reverse transcriptase–polymerase chain reaction (RT-PCR) for detection of influenza A viral nucleoprotein (8). All virus isolation attempts were conducted in a biosafety level-3 facility.

Genetic analyses and sequencing. RNA was extracted from influenza A virus-containing allantoic fluid by using a commercial kit (SV Total RNA Isolation system; Promega, Madison, WI). After reverse transcription, cDNAs were amplified by PCR with PyroStart™ Fast PCR Master mix (Fermentas, Foster City, CA). The identification of the HA-subtype of AI viruses was made using the primer sets as previously described (8). NA-subtyping was made by RT-PCR as described (15). Sequencing the HA gene of H5 and H7 subtypes was done using synthetic primers previously published (19). Template DNA was sequenced using BigDye Terminator version 3.1 (Applied Biosystems, Inc., Foster City, CA). Samples were analyzed using the Vector NTI 10.0 (Invitrogen, Carlsbad, CA) software package. MEGA 4 (Center for Evolutionary Functional Genomics, The Biodesign Institute, Tempe, AZ) was used to produce phylogenetic trees. For the comparison, HA gene sequences in the GenBank database were used to find the most closely related viruses and they were selected for inclusion in the phylogenetic trees.

Serologic test of isolates. The hemagglutination inhibition (HI) test was performed as previously described (4). Antigenic characterization of the influenza viruses was carried out by HI assay using seven chicken polyclonal antisera, as previously described (5). We also used the monoclonal antibodies CP62 and 364/I (courtesy of A. Klimov, Center for Disease Control and Prevention, Atlanta, GA), which were produced against HA of A/chicken/Pennsylvania/83 (H5N2) and react against H5-subtypes AI viruses in HI tests as previously described (22,26). The chicken antisera against the HA of influenza A corresponded to selected strains: A/swine/IA/30 (H1N1), A/duck/Ukraine/63 (H3N8), A/duck/HongKong/1264/97 (H4N6), A/goose/HongKong/437/99 (H5N2), A/Teal/HongKong/1312/97 (H6N2), A/chicken/Rostock/34 (H7N1), and A/duck/HongKong/1280/97 (H9N2; courtesy of R. G. Webster, St. Jude Children's Research Hospital, Memphis, TN). The HI assay started at a serum dilution of 1:40.

Intravenous pathogenicity test to chickens. Intravenous pathogenicity of isolates for chickens was determined according to guidelines established by the World Organisation of Animal Health (OIE) (12).

RESULTS AND DISCUSSION

A total of 606 samples of cloacal swabs were collected (Table 1) during wild bird surveillance in Crimea from March 2006 to November 2008 from bird species in the orders Anseriformes (n = 7), Columbiformes (n = 1), Gruiformes (n = 1), and Galliformes (n = 1). From these samples, 20 LPAI viruses were isolated in the first embryonating chicken egg passage from three different species of waterfowl (Table 1); mallard (n = 8), wigeon (n = 2), and garganey (n = 10). Nineteen AI isolates were recovered in the fall and one in the spring sampling periods. The most common subtypes were H4N6 (n = 6) and H3N8 (n = 4). The H3N6 (n = 3) and H3N1 (n = 2) subtypes were found only in 2008. H3N6 (n = 3) was found only in garganey and H3N1 (n = 2) was found in both

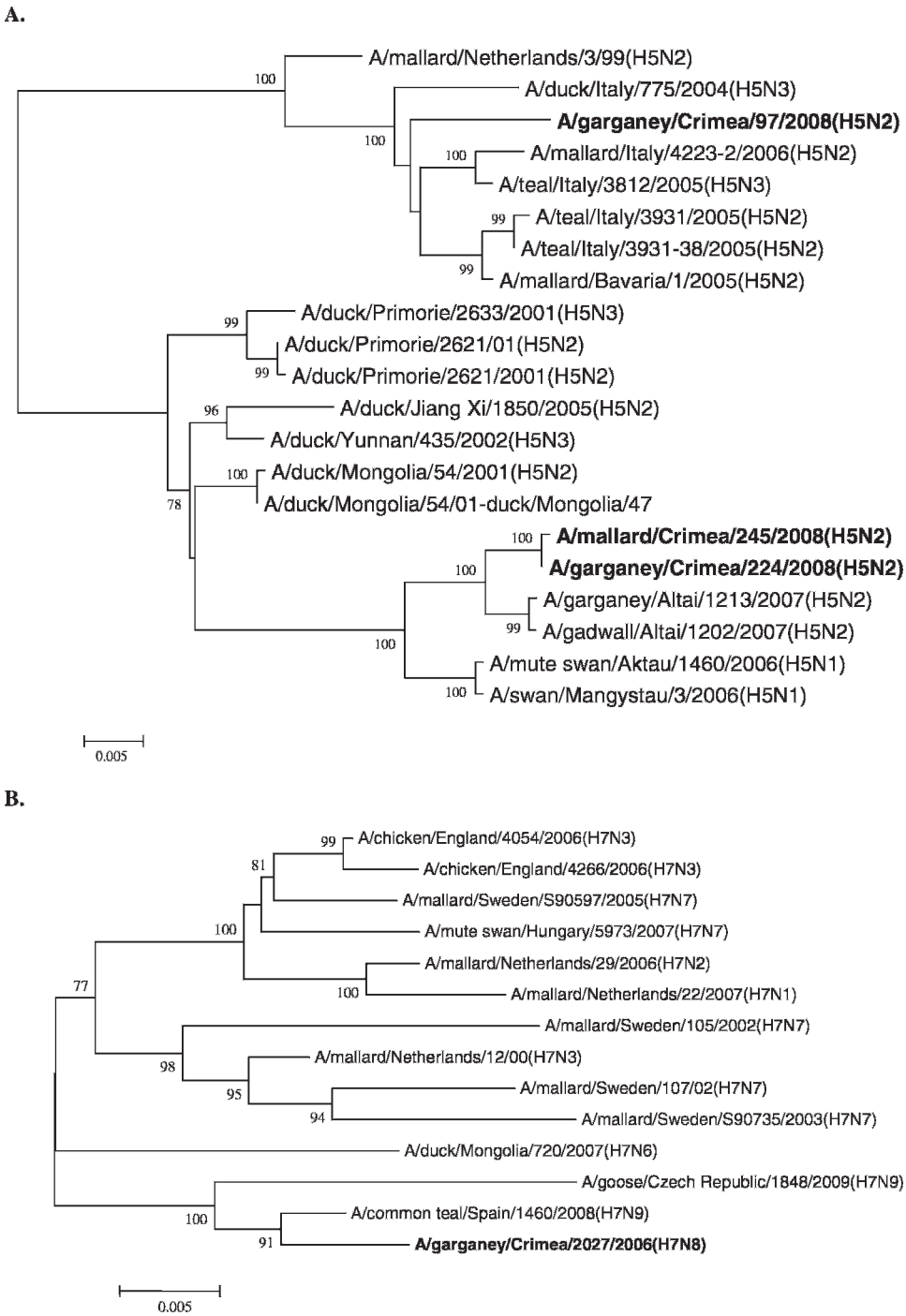

Fig. 1. Phylogenetic relationships of the HA gene of H5N2 AI viruses (A) and H7N8 (B) isolated in Crimea in 2008 with the most similar isolates included from GenBank. The tree was constructed by the neighbor-joining method. The topology of the tree was confirmed by 1000 bootstrapping steps. Analyses were conducted with nucleotide positions 1–1676 of the HA gene.

garganey and mallard. An H10N6 isolate was recovered from a garganey. An H7N8 was only recovered from a mallard, and H5N2 was recovered from a mallard ($n = 1$) and garganeys ($n = 2$). No H5 or H7 were recovered from wigeons. The only spring isolate was the H4N6 recovered from a wigeon. The higher frequency of H3 and H4 subtypes in our sample sets is in agreement with the higher frequency of H3 and H4 subtypes in North American dabbling ducks (6).

Based on chicken HA-typing antisera, three isolates of H5 and one of H7 HA subtypes were found. The three H5 isolates were further investigated in an HI test using monoclonal antibodies CP62 and 364/I, which reacted at a dilution of greater than 1:80,000 to all three of the isolates, confirming they were of the H5 subtype (22,26). Based on NA RT-PCR subtyping, the three H5 viruses were all N2 NA subtype and the H7 was an N8 NA subtype. No H5N1 subtype was detected in this survey; however, an H5N1 outbreak occurred in December, 2005 in Crimea with over 24,000 poultry deaths in the area (14).

The H5 and H7 viruses were pathotyped based on HA cleavage site sequence. The cleavage sites of H5N2 and H7N8 isolates were

PQRETR and PEIPKGR, respectively, which demonstrated them as being LPAI viruses (20). Furthermore, the intravenous pathogenicity index (IVPI) was determined for all 20 isolates in chickens according to guidelines established by OIE (12). None of the chickens had clinical signs, or died, over the 10-day experimental period, indicating that all isolates were of low pathogenicity for chickens (IVPI = 0.0).

Several of the HA and NA subtypes of LPAI virus in the current study have been previously identified in migratory bird species within various Asian flyways. The H4N6 and H3N8 subtypes have been recovered in Australia in 1984 and in New Zealand in 2002 (East Asia–Australian flyway) (10,13,18). H3N6 has been found since 2000 circulating in China and Mongolia (East Asia–Australian, Central Asia, and East Africa–West Asia flyways) (9,13,17), and H3N1 has been circulating in Italy (Black Sea–Mediterranean flyway) (11,13). H7N8 was previously found only on waterfowls in Japan during 1997–2000 (East Asia–Mediterranean flyway) (13,16). H5N2 has been reported in South Africa (Black Sea–Mediterranean and East Africa–West Asia flyways) (1,13), Italy (Black Sea–Mediterranean flyway) (2,13), and Korea (East Asia–Australian) (7,13). The geographic interconnection between the four European–Asian flyways, especially via summer breeding grounds, and the potential for AI viruses to reassort gene segments, can explain the historically reported genetic relatedness between AI viruses within Asia and Europe.

The full-length HA genes of the three H5N2 isolates, and one H7N8 isolate, were sequenced and submitted to GenBank (National Center for Biological Information, Bethesda, MD) under the following numbers: GU228593 (A/mallard/Crimea/224/2008 [H5N2]), GU228595 (A/garganey/Crimea/97/2008 [H5N2]), GU228594 (A/garganey/Crimea/2027/2006 [H7N8]), and GU228596 (A/mallard/Crimea/245/2008 [H5N2]). Phylogenetic analysis of the H5 HA gene showed that the A/mallard/Crimea/245/2008 (H5N2) and A/garganey/Crimea/224/2008 (H5N2) viruses were highly different from A/garganey/Crimea/97/2008 (H5N2; Fig. 1A). A/garganey/Crimea/97/2008 (H5N2) was genetically most similar to H5N2 and H5N3 AI viruses isolated in Italy during 2004–2006, whereas the other two isolates clustered with H5N2 AI viruses isolated in Altai Region, Western Siberia during 2007, and identity between the Crimea viruses was 99.1% at the nucleotide and 98.1% at the amino acid levels.

As represented in Fig. 1B, the A/garganey/Crimea/2027/2006 (H7N8) isolate was most closely related to a European LPAI virus, A/common teal/Spain/1460/2008 (H7N9). These strains exhibited, on average, 99.6% identity at the nucleotide level and 99.1% identity at the amino acid level. In addition, A/goose/Czech Republic/1848/2009 (H7N9) clustered in a sublineage with the Spanish and Crimean isolates and had 97.1% identity with the Crimean isolate at the nucleotide level.

The transmission of AI viruses, and their geographic spread, depends on the susceptibility of the hosts and on the flyways they travel during annual migration. In this study, the two H5N2 LPAI viruses isolated in Crimea were genetically most similar to other H5 LPAI viruses from Altai Region, Western Siberia isolated during the same time period (14). However, the other H5N2 LPAI virus and the H7N8 LPAI virus were most closely related to viruses in central and western Europe; H5 LPAI virus from Italy and H7 LPAI virus in Spain, respectively. Thus, for the first time, this demonstrates an ecologic connection between central and Western Europe with the Crimea region.

In conclusion, we note that our results emphasize the necessity to continue avian monitoring for a better understanding of the natural influenza A viruses cycle and underlines the importance of surveillance activities aimed at studying the circulation of virus strains with epidemiologic implications in domestic animals, human influenza, or both.

REFERENCES

1. Abolnik, C. Molecular characterization of H5N2 avian influenza viruses isolated from South African ostriches in 2006. Avian Dis. 51:873–879. 2007.

2. Cattoli, G., C. Terregino, V. Guberti, R. De Nardi, A. Drago, A. Salviato, S. Fassina, and I. Capua. Influenza virus surveillance in wild birds in Italy: results of laboratory investigations in 2003–2005. Avian Dis. 51(1 Suppl):414–416. 2007.

3. Chen, H., G. J. D. Smith, S. Y. Zhang, K. Qin, J. Wang, K. S. Li, R. G. Webster, J. S. M. Peiris, and Y. Guan. H5N1 virus outbreak in migratory waterfowl: a worrying development could help to spread this dangerous virus beyond its stronghold in Southeast Asia. Nature (London) 436:191–192. 2005.

4. Ellis, T. M., C. W. Leung, M. K. Chow, L. A. Bissett, W. Wong, Y. Guan, and J. S. M. Peiris. Vaccination of chickens against H5N1 avian influenza in the face of an outbreak interrupts virus transmission. Avian Pathol. 33:405–412. 2004.

5. Guan, Y., K. F. Shortridge, S. Krauss, P. S. Chin, K. C. Dyrting, T. M. Ellis, R. G. Webster, and M. Peiris. H9N2 influenza viruses possessing H5N1-like internal genomes continue to circulate in poultry in southeastern China. J. Virol. 74:9372–9380. 2000.

6. Krauss, S., C. A. Obert, J. Franks, D. Walker, K. Jones, P. Seiler, L. Niles, S. P. Pryor, J. C. Obenauer, C. W. Naeve, L. Widjaja, R. J. Webby, and R. G. Webster. Influenza in migratory birds and evidence of limited intercontinental virus exchange. PLoS Pathog. 3(11):e167. 2007.

7. Lee, J. H., P. N. Pascua, M. S. Song, Y. H. Baek, C. J. Kim, H. W. Choi, M. H. Sung, R. J. Webby, R. G. Webster, H. Poo, and Y. K. Choi. Isolation and genetic characterization of H5N2 influenza viruses from pigs in Korea. J. Virol. 83:4205–4215. 2009.

8. Lee, M. S., P. C. Chang, J. H. Shien, M. C. Cheng, and H. K. Shieh. Identification and subtyping of avian influenza viruses by reverse transcription-PCR. J. Virol. Methods 97:13–22. 2001.

9. Liu, M., S. He, D. Walker, N. Zhou, D. R. Perez, B. Mo, F. Li, X. Huang, R. G. Webster, and R. J. Webby. The influenza virus gene pool in a poultry market in Southcentral China. Virology 305:267–275. 2003.

10. Mackenzie, J. S., E. C. Edwards, R. M. Holmes, and V. S. Hinshaw. Isolation of ortho- and paramyxoviruses from wild birds in Western Australia, and the characterization of novel influenza A viruses. Aust. J. Exp. Biol. Med. Sci. 62(Pt. 1):89–99. 1984.

11. Moreno, A., I. Barbieri, E. Sozzi, A. Luppi, D. Lelli, G. Lombardi, M. G. Zanoni, and P. Cordioli. Novel swine influenza virus subtype H3N1 in Italy. Vet. Microbiol. 138:361–367. 2009.

12. OIE. Avian influenza. Terrestrial animal health code. Article 10.4.1. World Organisation for Animal Health, Paris. 2009.

13. Olsen, B., V. J. Munster, A. Wallensten, J. Waldenstrom, A. D. M. E. Osterhaus, and R. A. M. Fouchier. Global patterns of influenza A virus in wild birds. Science 312:384–388. 2006.

14. Onishchenko, G. G., S. P. Berezhnov, A. M. Shestopalov, A. Y. Alekseev, V. A. Ternovoy, A. B. Khaitovich, M. T. Krovyakova, S. V. Netesov, and I. G. Drozdov. Molecular-biologic analysis of avian influenza virus isolates, which caused epizootics on the south of West Siberia and in Crimea. Zhurnal Mikrobiologii Epidemiologii i Immunobiologii. 28–32. 2007.

15. Qiu, B. F., W. J. Liu, D. X. Peng, S. L. Hu, Y. H. Tang, and X. F. Liu. A reverse transcription-PCR for subtyping of the neuraminidase of avian influenza viruses. J. Virol. Methods 155:193–198. 2009.

16. Shengqing, Y., K. Shinya, K. Otsuki, H. Ito, and T. Ito. Isolation of myxoviruses from migratory waterfowls in San-in district, western Japan in winters of 1997–2000. J. Vet. Med. Sci. 64:1049–1052. 2002.

17. Spackman, E., D. E. Swayne, M. Gilbert, D. O. Joly, W. B. Karesh, D. L. Suarez, R. Sodnomdarjaa, P. Dulam, and C. Cardona. Characteriza-

tion of low pathogenicity avian influenza viruses isolated from wild birds in Mongolia 2005 through 2007. Virol. J. 6:190. 2009.

18. Stanislawek, W. L., C. R. Wilks, J. Meers, G. W. Horner, D. J. Alexander, R. J. Manvell, J. A. Kattenbelt, and A. R. Gould. Avian paramyxoviruses and influenza viruses isolated from mallard ducks (Anas platyrhynchos) in New Zealand. Arch. Virol. 147:1287–1302. 2002.

19. Suarez, D. L., M. L. Perdue, N. Cox, T. Rowe, C. Bender, J. Huang, and D. E. Swayne. Comparison of highly virulent H5N1 influenza A viruses isolated from humans and chickens from Hong Kong. J. Virol. 72:6678–6688. 1998.

20. Suarez, D. L., D. A. Senne, J. Banks, I. H. Brown, S. C. Essen, C. W. Lee, R. J. Manvell, C. Mathieu-Benson, V. Moreno, J. C. Pedersen, B. Panigrahy, H. Rojas, E. Spackman, and D. J. Alexander. Recombination resulting in virulence shift in avian influenza outbreak, Chile. Emerg. Infect. Dis. 10:693–699. 2004.

21. Swayne, D. E., and D. A. Halvorson. Influenza. In: Diseases of poultry, 12 ed. Y. M. Saif, J. R. Glisson, A. M. Fadly, L. R. McDougald, and L. Nolan, eds. Blackwell, Ames, IA. pp. 153–184. 2008.

22. To, K. F., P. K. S. Chan, K. F. Chan, W. K. Lee, W. Y. Lam, K. F. Wong, N. L. S. Tang, D. N. C. Tsang, R. Y. T. Sung, T. A. Buckley, J. S. Tam, and A. F. Cheng. Pathology of fatal human infection associated with avian influenza A H5N1 virus. J. Med. Virol. 63:242–246. 2001.

23. Webster, R. G., W. J. Bean, O. T. Gorman, T. M. Chambers, and Y. Kawaoka. Evolution and ecology of influenza A viruses. Microbiol. Rev. 56:152–179. 1992.

24. World Health Organization (WHO). Manual on animal influenza diagnosis and surveillance. 2002. [cited 2010 Jan 26]. Available from: http://www.who.int/csr/resources/publications/influenza/whocdscsrncs20025rev.pdf

25. WHO. Epidemic and pandemic alert and response (EPR). WHO manual on animal influenza diagnosis and surveillance [cited 2007 Feb 26]. Available from: http://www.who.int/vaccine_research/diseases/influenza/WHO_manual_on_animal-diagnosis_and_surveillance_2002_5.pdf

26. Yuen, K. Y., P. K. S. Chan, M. Peiris, D. N. C. Tsang, T. L. Que, K. F. Shortridge, P. T. Cheung, W. K. To, E. T. F. Ho, R. Sung, and A. F. B. Cheng. Members of the H5N1 study group. Clinical features and rapid viral diagnosis of human disease associated with avian influenza A H5N1 virus. Lancet 351:467–471. 1998.